Phytochemistry, 1976, Vol. 15, pp. 57–60. Pergamon Press Printed in England

REACTION MECHANISM OF GRAPE CATECHOL OXIDASE—A KINETIC STUDY

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(*Revised received* 7 *July* 1975)

Key Word Index—*Vitis vinifera*; Vitaceae; grape; catechol oxidase; 4-methylcatechol; kinetics

Abstract—The initial velocity of the oxidation of 4-methylcatechol by grape catechol oxidase was determined. The kinetic analysis indicates that first there is random binding of an oxygen and a 4-methylcatechol molecule to the enzyme. Then one product molecule is released prior to the binding of second 4-methylcatechol molecule which is followed by the release of a second product molecule. The true K_m values were determined; they were found to be 0·5 mM for oxygen and 17 mM for 4-methylcatechol.

INTRODUCTION

Knowledge concerning some of the fundamental properties of catechol oxidase is still very limited. A kinetic analysis of oxidation by the tea leaf enzyme by Gregory and Bendall [1], showed straight parallel double reciprocal plots when the enzyme was catalysing the oxidation of most substrates, including 4-methylcatechol under initial velocity conditions. Some kinetic parameters for the tyrosinase from mushrooms [2] and *Streptomyces* [3] have been reported, and sequential reaction mechanisms have been suggested on the basis of the kinetic analysis.

Bonner [4] has pointed out that the nature of the oxidation of monophenols, the cresolase activity and of *o*-diopherols, the catecholase activity of catechol oxidase has not been entirely clarified. It has been assumed that during the cresolase reaction, the monophenol is first hydroxylated to an *o*-diphenol and then further oxidized to an orthoquinone by a catecholase reaction. This was first suggested by Raper [5] because 3,4-dihydroxyphenylalanine (DOPA) could be isolated when tyrosine was incubated with tyrosinase and oxygen. Later Evans and Raper [6] showed that the rate of oxidation of DOPA by tyrosinase was much faster than the rate of oxidation of tyrosine. DOPA should therefore not accumulate in the reaction mixture. They concluded that another pathway exists which leads to the accumulation of DOPA. A reducing intermediate, 5,6-dihydroxy-dihydroindole-α-carboxylic acid was shown to take part in the reaction which reduces dopaquinone back to DOPA. Dressler and Dawson [7] and Kim and Tchen [8] published evidence that the cresolase reaction might not necessarily proceed through the formation of an *o*-dihydroxy derivative.

Kinetic studies would help to elucidate the mechanisms of these reactions. In this paper we report on a study of the kinetics of oxidation of 4-methylcatechol by grape catechol oxidase.

RESULTS AND DISCUSSION

In a previous paper [9] we showed that pre-treatment of chloroplasts with aqueous acetone solubilized catechol oxidase while causing little enzyme denaturation. The double reciprocal plots of velocity against substrate concentration obtained with enzyme solubilized in this manner were curved upwards, and the V_{max} values were not proportional to enzyme concentration. Enzyme activity versus oxygen concentration, in 0·1 M citrate buffer pH 5·0, showed Hill coefficients varying between 1·8 and 3·1. These characteristics could result from protein–protein interactions and enzyme conformational changes [10]. Such characteristics would readily be obtained in enzyme released from its membrane and make it unsuitable for kinetic analysis. We therefore decided to continue further kinetic studies using membrane bound-catechol oxidase as it occurs in the intact chloroplasts.

Freshly prepared chloroplasts from grapes in 0·5 M sucrose, containing 0·1 M sodium acetate buffer pH 4·5, showed Michaelis kinetics, and V_{max} values proportional to the chloroplast concentration. Velocities were calculated from the tangents of progress curves of change in oxygen concentration as a function of time, at various 4-methylcatechol (4 MC) concentrations, using freshly prepared chloroplasts. The experiments at each 4-methylcatechol concentration were carried out in triplicate. The triplicate values of the velocities at the various concentrations of the substrates were averaged and reciprocals calculated. Slopes, intercepts and correlation coefficients of the double reciprocal plots were obtained. These parameters were also calculated for the replots of slopes and intercepts in the same fashion (Tables 1 and 2, Figs. 1 and 2). The correlation coefficients obtained varied between 0·989 and 0·999. The slopes decreased progressively with the progressive increase of concentration of the fixed substrate indicating that the double reciprocal plots were straight intersecting lines. True K_m values were calculated from the slope/intercept of the replots of intercepts versus the reciprocal of the substrate concentration. The K_m value for oxygen was found to be 0·5 mM, and the K_m value for 4 MC was found to be 17 mM.

The stoichiometry of the catecholase reaction is assumed to be 2(4-methylcatechol) + O_2 $\rightleftharpoons$ 2(4-methylorthoquinone) + $2H_2O$ [11]. The quinone formed is

known to be unstable in the reaction medium. It undergoes further non-enzymatic reactions [12] which can lead to the consumption of an additional atom of oxygen per molecule of quinone. By applying the kinetic analysis of Cleland [13], it can be concluded from the linearity of the plots that an irreversible step occurs between the binding of the oxygen molecule and the binding of one of the two 4-methylcatechol molecules. The kinetics may be rendered more complicated, because the kinetics of the non-enzymatic reaction are superimposed on the kinetics of the enzymatic reaction. Nevertheless, the kinetical analysis is applicable if the enzymatic step is rate limiting.

The simplest mechanism which would show straight

Table 1. A. Slopes, intercepts and correlation coefficients, at various oxygen concentrations, of the double reciprocal plots of velocity vs 4-methylcatechol concentration of fresh grape chloroplasts catalysing the oxydation of 4-methylcatechol

Oxygen (mM)	Slope	Intercept	Correlation coefficient
0·060	0·227	0·0277	0·9975
0·072	0·201	0·0232	0·9988
0·084	0·174	0·0198	0·9973
0·096	0·146	0·0179	0·9977
0·108	0·142	0·0162	0·9976
0·120	0·139	0·0151	0·9992
0·132	0·131	0·0143	0·9979
0·144	0·124	0·0132	0·9984
0·156	0·122	0·0120	0·9989
0·168	0·114	0·0120	0·9982

B. Slopes, intercepts and correlation coefficients of the replots of the intercepts and the slopes vs the reciprocal of the oxygen concentration

	Slope	Intercept	Correlation coefficient
Replot of intercepts vs $1/O_2$	0·00142	0·00290	0·9981
Replot of slopes vs $1/O_2$	0·01047	0·05009	0·9891

The slope/intercept of the replot of the intercepts yields a K_m value for oxygen of 0·5 mM. Measurements were made in 0·5 M sucrose containing 0·1 M sodium acetate buffer pH 4·5 at 26°. Rates are in scale units/min. Calculations were made using linear regression.

Table 2. A. Slopes, intercepts and correlation coefficients at various 4-methylcatechol concentrations of the double reciprocal, plots of velocity vs oxygen concentration of fresh grape chloroplasts catalysing the oxidation of 4-methylcatechol

4-Methyl-catechol (mM)	Slope	Intercept	Correlation coefficient
2·5	0·00570	0·02331	0·9942
5·0	0·00336	0·01257	0·9917
7·5	0·00305	0·00834	0·9957
12·5	0·00226	0·00695	0·9959
25·0	0·00176	0·00636	0·9925
50·0	0·00179	0·00350	0·9985

B. Slopes, intercepts and correlation coefficients of the intercepts and the slopes vs the reciprocal of the 4-methylcatechol concentration

	Slope	Intercept	Correlation coefficient
Replots of intercepts vs 1/4MC	0·0499	0·00291	0·9920
Replots of slopes vs 1/4MC	0·0105	0·00146	0·9950

The slope/intercept of the replot of the intercepts yields a K_m value for 4-methylcatechol of 17 mM. Experimental conditions and calculations were as indicated in Table 1.

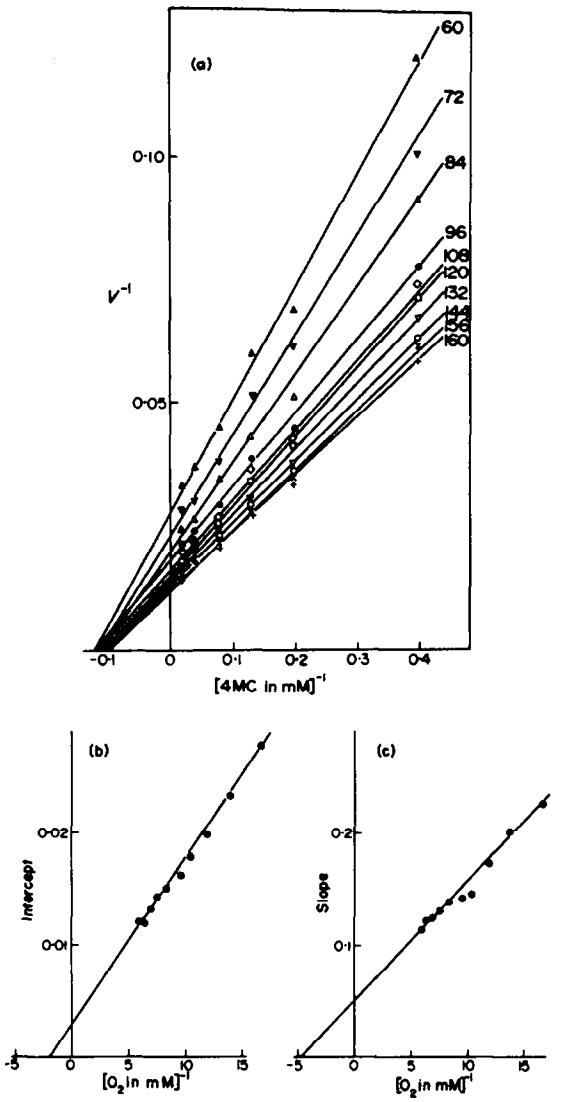

Fig. 1. A. Double reciprocal plots of velocity versus 4-methylcatechol concentration of fresh grape chloroplasts catalysing the oxidation of 4-methylcatechol. The numbers next to each line indicate oxygen concentration in micromolar. B. Replot of the intercepts vs the reciprocal of oxygen concentration. C. Replot of slopes vs the reciprocal of oxygen concentration. Experimental conditions and calculations as indicated in Table 1.

double reciprocal plots would be the following. One oxygen molecule and one of the two 4-methylcatechol molecules bind to the enzyme, leading to the release of one product molecule. Only after the release of the first product molecule would the second 4-methylcatechol molecule bind to the enzyme complex. This would be followed by the release of the second product molecule. Our results are similar to those of Gregory and Bendall [1] since in both cases there was an irreversible step between the binding of the two 4-methylcatechol molecules. It should however be remembered that there were considerable differences between the grape enzyme used here and the solubilised tea leaf enzyme used by Gregory & Bendall [1]. The latter enzyme was devoid of cresolase activity.

Duckworth & Coleman [2] and Lerch & Ettlinger [3] calculated kinetic constants by fitting their experimental data to bisubstrate rate equations. This can only give satisfactory results if the first sequence of the reaction is rate limiting. We therefore tested whether the reaction rates of the grape catechol oxidase fit Cleland's equation [13] for bireactant mechanisms:

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB}$$

where v is the initial velocity, A & B are substrate concentrations, V is V_{max} & K_a & K_b are Michaelis constants & K_{ia} & K_{ib} are substrates inhibition constants. We determined the K_i values for O_2 and 4-methylcatechol according to Cleland [13]. K_i for oxygen was found to be

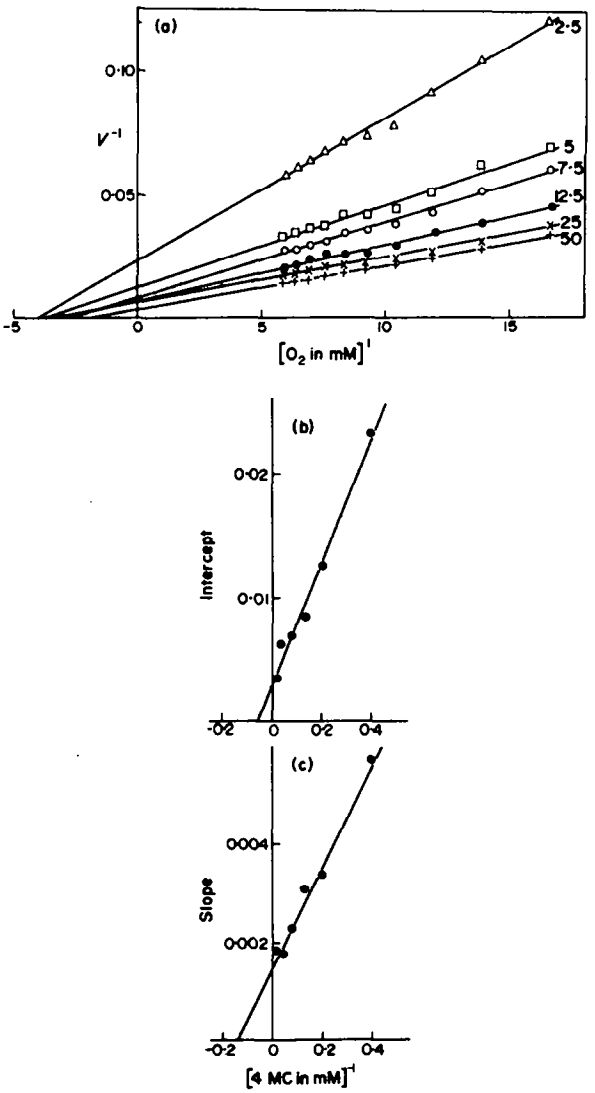

Fig. 2. A. Double reciprocal plots of velocity vs oxygen concentration of fresh grape chloroplasts catalysing the oxidation of 4-methylcatechol. The numbers next to each line indicate 4-methylcatechol concentrations in millimolar. B. Replot of intercept vs the reciprocal of 4-methylcatechol concentration. C. Replot of slope vs the reciprocal of 4-methylcatechol concentration. Experimental conditions and calculations were as indicated in Table 2.

0·21 mM and for 4-methylcatechol 7·37 mM. We compared the experimentally observed rates and those obtained by inserting the $K_i K_m$ & V_{max} values into the equation for bireactant mechanisms. In all cases observed and calculated values showed very good agreement, the average deviation being 2·5% from the experimentally observed rates. From this it is clear that the first sequence of the reaction must be rate limiting. If this were not so great divergence between calculated and observed values of v would have been obtained, as the catechol oxidase reaction involves 3 substrate molecules. As the reaction could fit a bireactant mechanisms $K_{ia}K_b$ & $K_{ib}K_a$ were calculated.

These were found to be $K_{iO_2}K_{4MC} = 3{\cdot}57\ \text{mM}^2$ and $K_{i4MC}K_{O_2} = 3{\cdot}67\ \text{mM}^2$. These values can only be the same in the reaction mechanism is a random binding of O_2 and 4-methylcatechol [13]. The binding of the substrates to catechol oxidase has been considered to be ordered [1] although random binding was regarded as possible [14]. The random binding can be reconciled with the known effect of the phenolic substrate on the K_m for oxygen. If the relaxation time of enzyme conformation induced by the phenolic substrate is slow compared to the time required for one cycle of catalysis then random binding would not prevent an effect of one substrate on the binding of the second one. Such slow relaxations have in fact been reported [15]. Alternatively a non-catalytic site for phenol, which controls the K_m for O_2 may be present.

We have also tried to study the kinetics of the cresolase reaction, but were not able to obtain interpretable data. Double reciprocal plots of velocity against oxygen concentration were S-shaped. Furthermore it is known from the literature that catechol oxidase undergoes inactivation during the catecholase reaction while it does not during the cresolase reaction [4]. In addition copper exchange is enhanced during the catecholase reaction but not during the cresolase reaction [7] and monophenol induces an enzyme conformational change [16] which considerably changes the enzyme's properties. It seems to us, therefore, likely that the mechanisms of the catecholase and the cresolase reactions differ.

EXPERIMENTAL

Fresh grape chloroplasts were prepared as previously described [9] except that buffer 0·5 M sucrose containing NaOAc 0·1 M pH 4·5 was used and that at all stages the chloroplasts were kept in buffered sucrose, pH 4·5. Enzyme activity was determined by measuring oxygen consumption using a Clark polarographic electrode according to Mayer *et al.* [17]. Calculations were made using an 8820A Hewlett-Packard calculator programmed for linear best fit by least squares. Commercial 4-methylcatechol was recrystallized from *n*-hexane.

Acknowledgement—We would like to thank Dr. J. Bar-Tana for his helpful comments and criticism and for the discussions.

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